



Lack of clastogenic effects of L-thyroxine in whole-blood cultured human lymphocytes

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Abstract

Thyroid hormones stimulate aerobic metabolism which may lead to oxidative stress accompanied by damage to various cellular macromolecules, including DNA. Previous comet assay studies have shown that thyroid hormones cause DNA damage due to the creation of reactive oxygen species (ROS). However, cytogenetic studies have been equivocal because although an increase in the sister-chromatid exchange frequency per cell has been reported increased micronuclei frequency has not. We used cytogenetic examination of chromosome breakage and aberrations in whole-blood cultures of human peripheral blood lymphocytes to investigate possible clastogenic effects when lymphocytes were exposed to 0.002 μ M to 50 μ M of L-thyroxine for 24 h and 48 h, these concentrations being chosen because they had been used in previous studies of sister-chromatid exchange and micronuclei frequency. Under our experimental conditions thyroxine did not induced any statistically significant increase in chromosome breakage or aberrations. This lack of clastogenic effects is in contrast to the reported comet assay results obtained using purified lymphocytes, possibly because whole-blood cultures contain catalase and glutathione peroxidase capable of reducing the effects of reactive oxygen species.

Key words: thyroxine, human lymphocytes, chromosome aberrations, mitotic index, ROS.

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Thyroid hormones stimulate development, growth and metabolism in most vertebrate tissues (Lazar, 2003). At the molecular level, increased metabolic rate and oxygen consumption is achieved both by the short-term action of 3,5-didodothyronine on mitochondrial cytochrome oxidase *c* and long-term changes of nuclear and mitochondrial gene transcription mediated by 3,3',5-triiodothyronine (Goglia *et al.*, 1999). The molecular mechanisms of thyroid hormone action have been thoroughly investigated but data is still sparse on possible genotoxic and mutagenic effects. Although it has been shown that thyroxine (T_4) acts as a co-carcinogen in the development of rat colon cancer (Iishi *et al.*, 1992) and that prolonged administration of thyroxine increases gastric carcinogenesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in Wistar rats (Iishi *et al.*, 1993), these effects resulted from non-genotoxic

mechanisms possibly due to the stimulation of cell proliferation. It is known, however, that thyroid hormones increase aerobic metabolism in mitochondria and causes intense production of reactive oxygen and nitrogen species, leading to the condition of oxidative stress (Venditti *et al.*, 2003; Fernandez *et al.*, 2005). Moreover, binding of thyroid hormones to specific nuclear receptors in target cells induces expression of enzymes related to redox processes and the total rate of oxygen consumption is enhanced (Oppenheimer *et al.*, 1996). Oxidative stress induced by thyroid hormones can lead to lipid and protein oxidation in rat liver cells (Tapia *et al.*, 1999). Experimentally induced hyperthyroidism in rat liver is accompanied by an increased rate of hepatic O_2 consumption, enhanced microsomal oxidative capacity and the generation of reactive oxygen species (ROS) (Fernandez *et al.*, 2003). In addition, treatment with thyroid hormone decreases the activity of superoxide dismutase (SOD), catalase and glutathione content in rat liver (Fernandez *et al.*, 1988). This depression of key antioxidative mechanisms favors oxidative stress in the liver (Tapia *et al.*, 1999).

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It is well established that oxidative stress causes damage to biological molecules such as proteins, membrane lipids and DNA. Oxidative DNA damage can produce a wide variety of DNA modifications including base and sugar lesions, strand breaks, DNA-protein cross-links and base-free sites (Evans *et al.*, 2004). Elevated levels of thyroid hormones can enhance production of ROS in mitochondria not only in organs under the strong influence of thyroid status (*i.e.* the liver), but also in human leukocytes (Magsino *et al.*, 2000). Indeed, more recent experimental findings have shown that thyroid hormones induce DNA damage in human lymphocytes (Djelic and Anderson, 2003) and sperm (Dobrzynska *et al.*, 2004), which can be observed in the comet assay. Since the antioxidant enzyme catalase reduced these effects, DNA damage was caused mainly by action of ROS. In our previous investigations, we also studied the cytogenetic effects of L-thyroxine on human peripheral blood lymphocytes by monitoring sister-chromatid exchange (SCE) and micronuclei as endpoints of the genotoxic exposure (Djelic *et al.*, 2006). However, thyroxine exhibited only weak genotoxic effects expressed as an increased frequency of SCE per cell in cultured human lymphocytes, whereas no effect was observed in the cytokinesis block micronucleus assay (Djelic *et al.*, 2006). Since these results of cytogenetic evaluation of thyroxine toxicity were equivocal, we undertook the investigation described in this paper to further elucidate possible clastogenic effects of thyroxine on cultured human lymphocytes.

The thyroid hormone L-thyroxine (3-[4-(4-Hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]-L-alanine (or T_4), molecular mass 776.87, CAS No. 51-48-9) was investigated at concentrations ranging from 0.002 μM to 50 μM (Table 1) dissolved in dimethyl sulfoxide (DMSO). In addition to untreated cultures, the negative control was 0.8% (v/v) DMSO (final conc. 0.1 M), without thyroxine, and the positive control was the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG, CAS No. 70-25-7), without DMSO, at a concentration of 1 μM . These chemicals were purchased from the Sigma Chemical Co., St. Louis, USA.

Human peripheral blood lymphocyte cultures were set up, as previously reported by Djelic *et al.* (1996), according to a slight modification of the protocol described by Evans and O'Riordan (1975). Briefly, 0.8 mL heparinized whole blood samples, obtained by venipuncture from three healthy Caucasian male volunteers under 30 years of age, were added to vials with 9.2 mL of RPMI 1640 media (Gibco, Grand Island, NY) supplemented with 15% (v/v) of heat-inactivated fetal calf serum (Gibco, Eggenstein, Germany) and supplemented with 1% (w/v) penicillin, 1% (w/v) streptomycin (Galenika, Belgrade, Serbia) and 5 $\mu\text{g mL}^{-1}$ of phytohemagglutinin (Murex Diagnostics Ltd., Dartford, England). Duplicate cultures from each donor were incubated in the dark for 72 h at 37 °C. The study was approved by the local Medical Ethics Commit-

tee, performed in accordance with the Declaration of Helsinki, and informed donor consent was also obtained.

Two L-thyroxine exposure times were used (24 h and 48 h), appropriate concentrations (0.002 μM to 50 μM , Table 1) of L-thyroxine dissolved in DMSO being added to the culture vials after 24 h incubation for the 48 h exposure experiment and at 48 h for the 24 h exposure experiment.

Two hours before harvesting 0.5 $\mu\text{g mL}^{-1}$ colcemid (Ciba, Basel, Switzerland) was added to the cultures and the cells treated with hypotonic 0.075 M KCl. After three standard cycles of fixation in methanol/acetic acid (3:1, v/v) the cell suspension was dropped onto chilled grease-free coded microscopic slides, air dried and stained in 5% (v/v) Giemsa (Merck, Darmstadt, Germany). We scored 200 mitotic plates per donor (100 per each duplicate culture) for each experimental concentration and for the positive and negative controls. Scoring of gaps and breaks was performed according to Brogger (1982) and the mitotic index (MI) was calculated as the percentage of cells in mitosis based on at least 2000 cells per donor.

Statistical analysis of cytogenetic endpoints and mitotic index was carried out using the chi-squared (χ^2) test (Sivikova *et al.*, 1999) at a significance level of $p = 0.05$ or lower.

In addition to the cytogenetic analysis we monitored possible cytotoxic or mitogenic effects of thyroxine using the analysis of the mitotic index on the same slides, the results for both types of analysis being shown in Table 1.

To evaluate the possible clastogenic effects of L-thyroxine we analyzed cultured whole-blood lymphocytes for chromosome lesions (gaps and breaks), rearrangements and numeric and structural aberrations, but we found no statistically significant structural chromosome aberrations after treatment with L-thyroxine dissolved in DMSO when compared to the negative control lymphocytes exposed to DMSO only, nor in comparison to untreated cultures, even though various types of chromosome lesions were detected and carefully scored by three independent assessors (Table 1). Thus, the overall percentage of aberrant cells in L-thyroxine treated cultures was in the range of 0% to 4.5% and there was no significant departure ($p > 0.05$) from the negative control base level. This agrees with our previous investigations (Djelic *et al.*, 2006) in which we observed no significant effect in the cytokinesis block micronucleus assay, using blood samples from the same donors and the same assay conditions. As expected, the positive control (MNNG) caused significant clastogenic effects, detectable as an increase in breaks and chromatid and isochromatid exchanges ($p < 0.001$), indicating that the experimental conditions were suitable for detecting clastogenic effects.

After 24 h exposure the mean mitotic index was 5.5% for the negative control lymphocytes from all three donors. The experimental mitotic index values for cultures exposed to L-thyroxine for 24 h ranged from 2.4% to 6.1%. The highest concentration (50 μM) of L-thyroxine caused a sig-

Table 1 - Analysis of chromosome gap and break frequencies and mitotic index in whole-blood cultures of human peripheral blood lymphocytes exposed *in vitro*, for 24 h and 48 h to different concentrations (μM) of L-thyroxine. The blood was taken from three healthy adult male donors and a total of 200 clear metaphase spreads from each donor were analyzed at each L-thyroxine concentration and for the positive and negative controls. The statistical analysis of breaks per metaphase did not include chromosome gaps (G), only chromatid breaks (CB), isochromatid breaks (IB), chromatid exchange (CE) and isochromatid exchange (IE). The mitotic index (MI) results were analyzed separately. In addition to untreated cultures, the negative control was 0.8% (v/v) dimethyl sulfoxide (DMSO) and the positive control was 1 μM N-methyl-L-N'-nitro-N-nitrosoguanidine (MNNG). Thyroxine has a molecular mass of 776.9 ($\mu\text{M} = 776.9 \mu\text{g L}^{-1}$) and the normal adult plasma total (bound + free) thyroxine level is about 5 $\mu\text{g dL}^{-1}$ to 12 $\mu\text{g dL}^{-1}$ (equivalent to 50 $\mu\text{g L}^{-1}$ to 120 $\mu\text{g L}^{-1}$ or 0.064 μM to 0.154 μM).

L-thyroxine concentration (μM) plus positive and negative controls	24 h exposure						48 h exposure					
	Types of chromosomal changes			Breaks per metaphase			Types of chromosomal changes			Breaks per metaphase		
	G	CB	IB	CE	IE	MI (%)	G	CB	IB	CE	IE	MI (%)
Donor 1												
L-thyroxine												
0.002	3	1				5.84	6	3	1			3.18
0.005	2	1				5.39	5	2	1	1		3.39
0.025	4	3				5.22	4	5				3.76
0.15	2		1			5.16	5	2				4.12
1.5		1				5.56	7	3	1			3.53
5	5	3				4.96	6	4	1			2.81
15	4	2				3.18 ^{&}	9	5				2.03 ^{&}
50	7	4				2.85 ^{&}	5	2	1	1		1.96 ^{&}
Untreated	5	3				5.98	7	4				4.04
Negative control (DMSO)	4	2				5.63	8	3				3.24
Positive control (MNNG)	14	9	5	2	3	2.12 ^{&}	20	17	6	2	4	1.82 ^{&}
Donor 2												
L-thyroxine												
0.002	3					5.47	6	6	2			3.86
0.005		2				6.07	4	2		1		4.08
0.025	6	1	1			5.93	3		2			3.73
0.15	4	3	2			5.78	8	4	1			3.80
1.5	5	1				5.40	6	4				4.15
5	4	2	1			5.02	6	3				3.42
15		2		1		4.63	5	2	1	1		2.18 ^{&}
50	6	2				3.22 ^{&}	10	3	2			1.97 ^{&}
Untreated	5	2				6.12	6	3	1			4.87
Negative control (DMSO)	6	4				5.78	7	5	1			4.25
Positive control (MNNG)	17	13	2	1	2	1.77 ^{&}	19	16	4	2	3	1.76 ^{&}
Donor 3												
L-thyroxine												
0.002	8	6				5.08	8	3	1			3.06
0.005	5					5.82	4	1				2.97
0.025	3	4				5.57	5	2		1		3.36
0.15	4	1				4.80	7	5				3.18
1.5	7	2				4.63	9	3	1	1		2.88
5	6	4	1	1		4.42	12	7		1		2.56
15	7	3	2			2.85 ^{&}	8	4		2		1.74 ^{&}
50	9	2		1		2.40 ^{&}	7	5	2	1		1.69 ^{&}
Untreated	6	2				5.30	9	5				3.32
Negative control (DMSO)	5	3				5.12	6	4				3.11
Positive control (MNNG)	22	19	2	4	4	2.03 ^{&}	13	21	4	2	2	1.71 ^{&}

[&]Statistically significant within the same donor and column compared to the negative control (chi-square test, $p < 0.01$).

[&]Statistically significant within the same donor and column compared to the negative control (chi-square test, $p < 0.001$).

nificant decrease in mitotic index compared to the value of the negative control. Furthermore, at concentrations of 15 μM the mitotic index was still significantly decreased for the lymphocytes from donor 1 ($p < 0.01$) and donor 3 ($p < 0.001$), whereas for donor 2 although there was a decrease in the mitotic index at this concentration the decrease was not significant. After 48 h exposure the mean mitotic index for the negative control lymphocytes from the three donors was 3.5%, whereas the overall range in cultures treated with L-thyroxine was from 1.7% to 4.1%. The two highest L-thyroxine concentrations (15 μM and 50 μM) caused a significant decrease of mitotic index in the lymphocytes from all three donors at 48 h (Table 1). However, these concentrations far exceed not only the normal blood thyroxine level ($\sim 0.1 \mu\text{M}$) and level in L-thyroxine treated patients, but also L-thyroxine level both in severe hyperthyroidism (Ganong, 1995) and acute L-thyroxine overdose (Berkner *et al.*, 1991). As expected, the positive control significantly lowered the mitotic index values for the lymphocytes from all three donors, both after 24 h and 48 h of exposure (Tables 1).

Investigations in the field of genetic toxicology have mainly concentrated on environmental pollutants, most of which result from intense development of chemical and other industries (Pitarque *et al.*, 2002; Stanimirovic *et al.*, 2005). However, there is increasing evidence that substances which are normally present in human or animal bodies may, under the certain circumstances, exhibit deleterious effects on genetic material and therefore act as endogenous mutagenic agents (Lutz, 1990; Totsuka *et al.*, 2005).

One of the best studied group of endogenous mutagenic agents are estrogenic hormones (Anderson *et al.*, 2003; Schallreuter *et al.*, 2006). It is well established that metabolic conversion of phenolic estrogen moieties accompanied by the generation of ROS leads to covalent damage of cellular macromolecules including DNA (Liehr, 2001; Li *et al.*, 2004). Interestingly, there are some experimental indications that phenolic groups of some non-steroid hormones (adrenaline, thyroid hormones) and neurotransmitters (dopamine, noradrenaline) may undergo redox cycling accompanied by the generation of ROS which favor oxidative stress (Moldeus *et al.*, 1983; Djelic and Anderson, 2003; Dobrzynska *et al.*, 2004). It thus seems that oxidative stress is the key mechanism of the genotoxic effects of both steroid and non-steroid hormones with phenolic groups.

The comet assay has shown that thyroid hormones can cause *in vitro* DNA damage to purified human lymphocytes (Djelic and Anderson, 2003) and sperm (Dobrzynska *et al.*, 2004) and that the damage can be reduced by the antioxidant enzyme catalase, indicating that the genotoxic effects of thyroid hormones may be principally due to ROS. The suggestion that L-thyroxine can have genotoxic effects is supported by reports of increased SCE in human lymphocytes treated with relatively high concentrations of L-thy-

roxine, although since L-thyroxine did not induce micronuclei under the same experimental conditions the cytogenetic effects of L-thyroxine were unclear (Djelic *et al.*, 2006). It is important to note, however, that in the study by Djelic and Anderson (2003) the DNA damage attributed to thyroid hormones was detected by the comet assay of purified human lymphocytes, with the cytogenetic evaluation of the genotoxic effects of L-thyroxine on lymphocytes from whole blood cultures producing equivocal results. These discrepancies may be explained, at least in part, by the findings of Andreoli *et al.* (1999) who reported more severe genotoxic effects on purified lymphocytes than compared to lymphocytes from whole-blood cultures, probably because erythrocytes in whole blood contain catalase and glutathione peroxidase which eliminate hydrogen peroxide and reduce the amount of ROS available for generating DNA damage.

Our results show that L-thyroxine did not produce clastogenic effects which could be detected on the basis of cytogenetic analysis and supports previous observations that L-thyroxine does not cause any significant increase in the micronucleus frequency of human peripheral blood lymphocytes (Djelic *et al.*, 2006). However, it should be pointed out that thyroid hormones caused DNA damage in the comet assay, at high concentrations in a range 10–100 μM (Djelic and Anderson, 2003; Dobrzynska *et al.*, 2004). Although these concentrations are comparable with two highest concentrations of L-thyroxine in the present study, genotoxic effects were observed only in the comet assay, which is more sensitive in comparison to the cytogenetic analysis.

Analysis of clastogenic effects has not revealed any significant difference between untreated cultures and negative controls treated with DMSO (Table 1), as observed previously by de Lima *et al.* (2005). Also, there was no significant response in thyroxine-treated cultures, in comparison to both untreated and DMSO treated cultures.

The percentage of cells in mitosis was significantly decreased only at two highest L-thyroxine concentrations, possibly due to cytotoxic effects. This finding is in accordance with our previous cytogenetic study (Djelic *et al.*, 2006) in which we observed decreases in mitotic index at the same experimental concentrations of L-thyroxine. As expected, positive control caused a more profound decrease in mitotic index, possibly due to arrested mitosis as a result of the repair of genetic damage or cytotoxic effects due to relatively high levels of genetic damage.

The results indicate that normal levels of L-thyroxine do not produce clastogenic effects on human lymphocytes in whole-blood cultures. In this investigation, the concentration of L-thyroxine of 0.15 μM corresponds to the total plasma level in euthyroid persons (Ganong, 1995). The higher concentration of 1.5 μM exceeds the total serum L-thyroxine concentration in Graves disease, but it is comparable to L-thyroxine blood level in an acute overdose

(Berkner *et al.*, 1991). Although L-thyroxine can increase the SCE rate (Djelic *et al.* 2006), the molecular mechanisms of SCE formation and their biological significance are not clear. Furthermore, the SCE test only indicates exposure to genotoxins, while the chromosome aberration and micronucleus assays are mutagenicity tests (Speit and Henderson, 2005) and are therefore of more significance than the SCE test. Based on these results we conclude that, under the experimental conditions employed in this study, L-thyroxine does not exhibit cytogenetically detectable genotoxic effects in lymphocytes from whole-blood cultures.

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